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Multidrug Efflux Pumps and Antimicrobial Resistance in *Pseudomonas aeruginosa* and Related Organisms

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Abstract

Pseudomonas aeruginosa **is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobial agents. A major contribution to this intrinsic multidrug resistance is provided by a number of broadly-specific multidrug efflux systems, including MexAB-OprM and MexXY-OprM. In addition, these and two additional tripartite efflux systems, MexCD-OprJ and MexEF-OprN, promote acquired multidrug resistance as a result of mutational hyperexpression of the efflux genes. In addition to antibiotics, these pumps promote export of numerous dyes, detergents, inhibitors, disinfectants, organic solvents and homoserine lactones involved in quorum sensing. The efflux pump proteins are highly homologous and consist of a cytoplasmic membrane-associated drug-proton antiporter of the Resistance-Nodulation-Division (RND) family, an outer membrane channel-forming protein [sometimes called outer membrane factor (OMF)] and a periplasmic membrane fusion protein (MFP). Homologues of these systems have been described in** ***Stenotrophomonas maltophilia*****,** ***Burkholderia cepacia*****,** ***Burkholderia pseudomallei*** **and the non-pathogen** ***Pseudomonas putida*****, where they play a role in export of and resistance to multiple antimicrobial agents and/or organic solvents. Although the natural function of these multidrug efflux systems is largely unknown, their contribution to antibiotic resistance and their conservation in a number of important human pathogens makes them logical targets for therapeutic intervention.**

Introduction

The continued over reliance on and imprudent use of antimicrobials has tended to enrich for pathogens that are intrinsically antibiotic resistant or have acquired resistance mechanisms. Organisms such as *P. aeruginosa*, *S. maltophilia* and *Burkholderia* spp. are of increasing clinical importance because of their innate resistance to multiple agents and their ability to develop high-level multidrug resistance (MDR). Perhaps not surprisingly, this resistance owes much to the presence of broadly-specific efflux systems which export and, thus, provide resistance to multiple antimicrobials. Antimicrobial efflux systems have been grouped into 5 families, although the major multidrug efflux systems responsible for intrinsic and acquired MDR in the aforementioned organisms utilize a drug-proton antiporter of the RND family (Saier *et al.*, 1994; Nikaido, 1998) (Table 1). This cytoplasmic membrane component typically functions with auxiliary proteins present in the outer membrane (the channel-forming OMF) and the periplasm (the MFP which couples the cytoplasmic membrane transporter to the OMF) (Table 1) to promote drug extrusion across both membranes simultaneously.

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Pseudomonas aeruginosa

P. aeruginosa is an opportunistic human pathogen associated with nosocomial infections of individuals immunocompromised as a result of burns or other severe trauma, underlying diseases, including cancer, diabetes and cystic fibrosis (CF), deliberate immunosuppression and major surgery (Botzenhart and Döring, 1993). The organism's intrinsic resistance has long been attributed to the outer membrane, a barrier of limited permeability (Nikaido, 1989). While the reduced uptake likely does limit access of antimicrobials to their targets within the cells, this is dependent upon additional resistance mechanisms such as drug efflux (Poole *et al.*, 1993b; Ma *et al.*, 1994). Indeed, it is now clear that the intrinsic multidrug resistance of this organism results from the synergy between outer membrane impermeability and chromosomally-encoded multidrug efflux pumps of the RND-MFP-OMF type (Germ *et al.*, 1999; Li *et al.*, 2000b). To date, 4 RND-MFP-OMF type MDR efflux systems have been described in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Homologues of these have also been reported in a number of Gram-negative organisms, where they play an equally important role in intrinsic and acquired resistance to antimicrobials (Poole, 2000). Recently, a defective version of the QacE multidrug exporter of the Small Multidrug Resistance (SMR) family (Paulsen *et al.*, 1996), QacEΔ1 (Paulsen *et al.*, 1993), was reported in a single *P. aeruginosa* isolate (Kucken *et al.*, 2000), although no correlation with resistance to intercalating dyes and quaternary ammonium compounds was seen.

MexAB-OprM

The first *P. aeruginosa* multidrug efflux system to be described, MexAB-OprM (Poole *et al.*, 1993a; Poole *et al.*, 1993b; Gotoh *et al.*, 1995; Li *et al.*, 1995) is expressed constitutively in cells grown in standard laboratory media, where it contributes to intrinsic resistance to a number of antimicrobials including fluoroquinolones, β-lactams, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim and sulphonamides (Köhler *et al.*, 1996; Poole

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Table 1. Multidrug efflux systems of *B. cepacia*, *B. pseudomallei*, *P. aeruginosa*, *P. putida* and *S. maltophilia*

Organism	Efflux components			Regulatory gene(s)	Expression[a]	Substrates[d]	References[e]	Accession number(s)
	MFP	RND	OEP					
Burkholderia cepacia	CeoA	CeoB	OpcM	?	wt/?;mutant/+	CM,CP,TP	(Burns *et al.*, 1989; Burns *et al.*, 1996)	U97042, U38944
Burkholderia pseudomallei	AmrA	AmrB	OprA	*amrR*	wt/+	ML,AG	(Moore *et al.*, 1999)	AF072887
Pseudomonas aeruginosa	MexA	MexB	OprM	*mexR*	wt/+; *nalB*/+++; *nalC*[b]/++	BL,FQ,CM,TC,NV,TP, SM,ML,EB,AC,CV,SDS, AH,HL,CL,TL,IR,TS	(Poole *et al.*, 1993a; Poole *et al.*, 1993b; Gotoh *et al.*, 1995; Li *et al.*, 1995)	L11616, L23839
	MexC	MexD	OprJ	*nfxB*	wt/-; *nfxB*/++	BL,FQ,CM,TC,NV,TP,ML, CV,EB,AC,SDS,AH,CL,TS	(Poole *et al.*, 1996)	U57969
	MexE	MexF	OprN	*mexT*	wt/-; *nfxC*[b]/++	FQ,CM,TP,AH,TS	(Köhler *et al.*, 1997)	AJ007825, X99514
	MexX (AmrA)	MexY (AmrB)	OprM	*mexZ* (*amrR*)	wt/+	FQ,AG,TC,ER	(Mine *et al.*,1999; Aires *et al.*,1999; Westbrock-Wadman *et al.*, 1999)	AB015853, AF147719
	pa1435[f]	pa1436[f]	?[g]	?	?	?	(Stover *et al.*, 2000)	AAG04824, AAG04825
	pa3677[f]	pa3676[f]	?	?	?	?	(Stover *et al.*, 2000)	AAG07076, AAG07064
	pa4374[f]	pa4375[f]	?	?	?	?	(Stover *et al.*, 2000)	AAG07762, AAG07763
	pa3523[f]	pa3522[f]	pa3521[f]	?	?	?	(Stover *et al.*, 2000)	AAG06911, AAG06910, AAG06909
	pa4206[f]	pa4207[f]	pa4208[f]	?	?	?	(Stover *et al.*, 2000)	AAG07593, AAG07594, AAG07595
Pseudomonas putida	MepA	MepB	MepC	*mepR*	wt/-; mutant/+	BL,TC,NV,ER,AH	(Fukumori *et al.*, 1998)	—[h]
	SrpA	SrpB	SrpC	?	wt/inducible[c]	AH	(Kieboom *et al.*, 1998)	AF029405
	TtgA	TtgB	TtgC	?	wt/?; mutant/+	CM,AP,TC,TO	(Ramos *et al.*, 1998)	AF031417
	TtgD	TtgE	TtgF	?	wt/inducible[c]	AH	(Mosqueda and Ramos, 2000)	Y19106
Stenotrophomonas maltophilia	SmeA	SmeB	SmeC	*smeRS*	?	BL,AG,FQ	(L. Zhang, X.-Z. Li, and K. Poole, unpublished)	AF173226
	SmeD	SmeE	SmeF	?	wt?/mutant +	TC,ER,FQ,EB	(Alonso and Martinez, 2000)	AJ252200

[a]Relative expression of the corresponding efflux system is indicted in wild type (wt) cells (where known) and in mutants known to express the system. In instances where the nature of the mutation leading to enhanced efflux gene expression is known, the gene is indicated along with the relative level of gene expression. wt/+ mutant/++, efflux system is expressed in wt cells (under laboratory growth conditions) but expression is enhanced in resistant strains; wt/- mutant/+, efflux system is not expressed in wt cells but is expressed in resistant strains; ?, expression status unknown.
[b]The *nalC* and *nfxC* genes have not yet been identified.
[c]Efflux system is inducible by the aromatic hydrocarbons that are substrates for efflux.
[d]AG, aminoglycosides, BL, β-lactams, FQ, fluoroquinolones, CM, chloramphenicol, TC, tetracycline, NV, novobiocin, TP, trimethorpim, SM, sulphonamides, ML, macrolides, CP, ciprofloxacin, ER, erythromycin, AP, ampicillin, EB, ethidium bromide, AC, acriflavine, CV, crystal violet, SDS, sodium dodecyl sulphate, AH, aromatic hydrocarbons, TO, toluene, HL, homoserine lactones, CL, cerulenin, TL, thiolactomycin, IR, irgasan, TS, triclosan. In instances where only one member of a class of antimicrobial has been tested or is known to be a substrate for a given pump, that member is identified. Where several members of an antimicrobial class are known to be substrates, the class is identified rather than the actual compounds tested.
[e]Only references describing the original characterization and/or sequencing of the efflux genes are highlighted
[f]Identified by a BLAST search of the recently completed *P. aeruginosa* genome sequence. The role, if any, of these in multidrug efflux remains to be determined.
[g]Unknown
[h]no accession number

et al., 1996b; Srikumar *et al.*, 1997; Srikumar *et al.*, 1998; Li *et al.*, 1998b). Given the conservation of the *oprM* gene in all known serotypes of *P. aeruginosa* (Bianco *et al.*, 1997), this efflux system may well play an essential role in the intrinsic antimicrobial resistance of all examples of this organism. The MexAB-OprM system is also hyperexpressed in in vitro-selected (Rella and Haas, 1982; Masuda and Ohya, 1992; Masuda *et al.*, 1995; Köhler *et al.*, 1996; Poole *et al.*, 1996b) and clinical (Piddock *et al.*, 1992; Jalal and Wretlind, 1998; Ziha-Zarifi *et al.*, 1999; Jalal *et al.*, 2000) *nalB* [or cfxB (Celesk and Robillard, 1989)] mutants, which are highly resistant to these agents. Although such resistance was typically seen in planktonic cells, MexAB-OprM may also be important for the resistance of biofilm-grown *P. aeruginosa* to certain antimicrobials (Brooun *et al.*, 2000). Though often selected by fluoroquinolones in vitro and in vivo, *nalB* isolates have also been identified amongst tetracycline (Hamzehpour *et al.*, 1995; Jalal *et al.*, 1999; Alonso *et al.*, 1999) and chloramphenicol resistant strains (Jalal *et al.*, 1999). Indeed, in a recent study invoking directed mutagenesis in the development of tetracycline resistance, the majority of tetracycline-resistant strains obtained were *nalB* mutants (Alonso *et al.*, 1999). In addition to medically-relevant antimicrobials, MexAB-OprM also exports a variety of dyes and detergents (Srikumar *et al.*, 1997; Srikumar and Poole, 1999), inhibitors of fatty acid biosynthesis (Schweizer, 1998), organic solvents (Li *et al.*, 1998a; Li and Poole, 1999) and homoserine lactones associated with quorum sensing (Evans *et al.*, 1998; Pearson *et al.*, 1999). The observation that solvents are pump substrates is consistent with a report that a number of solvent tolerant mutants of *P. aeruginosa* hyperexpress MexAB-OprM (Li and Poole, 1999).

A gene, *mexR*, occurs upstream of the efflux genes (Poole *et al.*, 1996b) and is the target of mutations in *nalB* strains (Poole *et al.*, 1996b; Jalal and Wretlind, 1998; Ziha-Zarifi *et al.*, 1999; Saito *et al.*, 1999; Srikumar *et al.*, 2000). A repressor (Srikumar *et al.*, 2000), MexR negatively regulates expression of *mexAB-oprM* (Poole *et al.*, 1996b; Srikumar *et al.*, 2000) and is responsible for negative autoregulation of *mexR* (Poole *et al.*, 1996b). MexR is a member of the MarR family of regulators (Miller and Sulavik, 1996). MarR, the product of the first gene of the *marRAB* operon (also called the *mar* locus) in *E. coli*, negatively

regulates *marRAB* expression, thereby controlling expression of the transcriptional activator MarA and, thus, several MarA-regulated genes associated with resistance including the *acrAB* multidrug efflux operon (Alekshun and Levy, 1999). Recently, the MexR protein was purified to homogeneity and shown to bind as a dimer to two sites in the *mexR-mexA* intragenic region, near *mexR* and overlapping promoters for *mexR* and *mexAB-oprM* (Evans *et al.,* 2001). The MexR operator sequence (*mexO*) is an inverted repeat, reminiscent of the inverted repeat of *marO*, which also binds a MarR dimer (Martin and Rosner, 1995).

MexAB-OprM hyperexpression also occurs independently of mutations in *mexR* or the *mexR* and *mexAB-oprM* promoter regions (Ziha-Zarifi *et al*., 1999; Srikumar *et al*., 2000). These *nalC* mutants (Srikumar *et al*., 2000) presumably carry a mutation in an unidentified regulator of *mexAB-oprM* expression. Growth phase regulation of MexAB-OprM has been reported in wild type and *nalB* strains of *P. aeruginosa*, with expression increasing throughout growth and reaching a maximum in late log phase (Evans and Poole, 1999). The gene(s) responsible, too, have yet to be identified although it is clear from these studies that regulation of *mexAB-oprM* is rather complex.

Topological analysis of MexB (Guan *et al*., 1999) and OprM (Wong and Hancock, 2000) has revealed that these integral membrane proteins span their respective membrane several times. MexB possesses 12 membrane-spanning helices with two very pronounced periplasmic loops between helices 1 and 2, and helices 7 and 8 (Guan *et al*., 1999) that likely function in interactions with MexA and/or OprM. OprM is predicted to have 16 membrane-spanning β-strands (Wong and Hancock, 2000), typical of porins, although the decided lack of similarity between the porin family and OMFs (Johnson and Church, 1999) suggests that a porin model may not be apt for OprM. Neural network prediction (Diederichs *et al*., 1998) of OprM topology suggests that OprM is substantially periplasmic (A. Ferguson, personal communication) in contrast with the model proposed by Wong and Hancock (Wong and Hancock, 2000). In light of the recent crystal structure of TolC (Koronakis *et al*., 2000), a homologue of OprM, the proposed model is likely incorrect and, indeed, two recent reports support a TolC-like structure for OprM (Li and Poole, 2001; Wong *et al*., 2001). The TolC channel is a novel structure, existing as a trimer and spanning both the outer membrane (as a β –barrel comprised of 12 membrane-spanning segments, 4 from each monomer) and periplasm (as a α-helical barrel) (Koronakis *et al*., 2000). Measuring 140 Å in length, the channel is open at the distal (extracellular) end and tapers almost to a close at the proximal (periplasmic) end, with an internal diameter of 35 Å for much of its length (Koronakis *et al*., 2000). It is likely, therefore, that OprM and TolC interact directly with their corresponding RND components in promoting drug export across both the periplasm and outer membrane. Channel-forming activity has been demonstrated for purified OprM, although the channel size observed was likely less than that required to accommodate the various known substrates of MexAB-OprM (Wong and Hancock, 2000). The probable involvement of the TonB energy-coupling protein in MexAB-OprM-mediated drug efflux and resistance (Zhao *et al*., 1998a) suggests, however, that TonB-promoted conformational changes in OprM may be necessary for full channel opening and drug extrusion across the outer membrane.

Examination of the OprM amino acid sequence reveals the presence of a lipoprotein box, typically the site of acylation of lipoproteins, at the N-terminus and, indeed, fatty acylation of OprM has been demonstrated (Nakajima *et al*., 2000) (N. Bianco and K. Poole, unpublished). Still, abrogation of acylation by mutation of OprM yields a functional OprM protein (Li and Poole, 2001; Nakajima *et al*., 2000). Given that TolC, the OMF of the AcrAB-TolC MDR efflux system of *E. coli* is itself not a lipoprotein (Hackett and Reeves, 1983), the significance of OprM acylation is certainly in doubt. The MexA MFP is also a lipoprotein although acylation of this protein, too, is dispensable for function (Yoneyama *et al*., 2000). This is intriguing since, unlike OprM, which is an integral membrane protein whose association with the outer membrane is assured, acylation or no, MexA is periplasmic protein apparently anchored to the cytoplasmic membrane via its lipid tail (Yoneyama *et al*., 2000). Perhaps, a cytoplasmic membrane association is only necessary to facilitate an interaction with the MexB component. Under conditions where an unacylated MexA is hyperexpressed (from a multicopy plasmid-borne gene [Yoneyama *et al*., 2000]), sufficient MexA-MexB complexes may form to promote a wild type level of efflux activity despite the lack of membrane anchoring.

The contribution of MexAB-OprM to β-lactam efflux and resistance is interesting, both because reports of efflux of this class of compound have been comparatively rare and because β-lactams act on periplasmic rather than cytoplasmic targets, in contrast to all other MexAB-OprM antibiotic substrates. This lead to an initial suggestion that OprM may dictate β-lactam specificity, as efflux across the outer membrane would be sufficient for resistance. Still, OprM alone was insufficient for β-lactams resistance (Wong *et al*., 1997), and while swapping outer membrane proteins between the MexCD-OprJ and MexAB-OprM systems yielded functional chimeric efflux systems, β-lactam resistance was dependent upon MexAB and not OprM (Srikumar *et al*., 1997). The MexAB-OprM contribution to β-lactam resistance varies with the β-lactam examined, in some instances playing a more important role than the chromosomally-encoded β-lactamase of *P. aeruginosa* (Nakae *et al*., 1999; Masuda *et al*., 1999). Moreover, loss of MexAB-OprM was shown to compromise, to some extent, the β-lactam resistance of β-lactamase derepressed and penicillin-binding protein mutants (Srikumar *et al*., 1999), highlighting its significance to the net resistance of these mutants.

Of the β-lactams, only carbapenems appear to be poor substrates for MexAB-OprM. Nonetheless, expression of this efflux system is correlated with resistance to one carbapenem, meropenem (Masuda and Ohya, 1992; Li *et al*., 1994; Köhler *et al*., 1999b). It has been suggested that this may be due to the need for efflux systems like MexAB-OprM to access their substrates within the cytoplasmic membrane (Nikaido, 1996; Ocaktan *et al*., 1997), inasmuch as meropenem is much more amphiphilic than those carbapenems that appear not to be substrates for MexAB-

OprM (Köhler *et al.*, 1999b). In any case, OprD-deficiency (see below) and not MexAB-OprM hyperexpression is the predominant resistance mechanism for carbapenems such as meropenem, with MexAB-OprM hyperexpression only seen in highly meropenem-resistant derivatives of previously OprD-deficient parents (Köhler *et al.*, 1999b).

MexCD-OprJ

The MexCD-OprJ MDR efflux system (Poole *et al.*, 1996a) is not detectable in wild type cells, at least under usual laboratory growth conditions (Hosaka *et al.*, 1995; Srikumar *et al.*, 1997). Expression of the efflux system is seen, however, in *nfxB* MDR mutant strains isolated in vitro (Hirai *et al.*, 1987; Masuda *et al.*, 1995; Poole *et al.*, 1996a) and in the clinic (Jakics *et al.*, 1992; Yoshida *et al.*, 1994; Jalal and Wretlind, 1998; Jalal *et al.*, 2000), as a result of mutations in the *nfxB* gene (Okazaki *et al.*, 1991; Okazaki and Hirai, 1992). Occurring immediately upstream of the *mexCD-oprJ* genes, *nfxB* encodes a repressor of *mexCD-oprJ* (Poole *et al.*, 1996a) and *nfxB* (Shiba *et al.*, 1995) expression (Poole *et al.*, 1996a), and MexCD-OprJ hyperexpression in *nfxB* mutants, thus, results from depression of the efflux genes. Originally identified as a determinant of fluoroquinolone resistance (Hirai *et al.*, 1987), MexCD-OprJ accommodates a variety of antimicrobial agents including macrolides, chloramphenicol, novobiocin, tetracycline, trimethorpim and some β-lactams (Masuda *et al.*, 1996; Köhler *et al.*, 1996; Srikumar *et al.*, 1997; Gotoh *et al.*, 1998b). Although the MexCD-OprJ export of β-lactams was originally reported to be limited to 4th generation cephems (e.g. cefpirome and cefepime) (Masuda *et al.*, 1996; Poole *et al.*, 1996a), more recent studies using mutants lacking MexAB-OprM but expressing MexCD-OprJ have confirmed the ability of this efflux system to accommodate ordinary cephems such as cefoperazone and ceftazidime (Srikumar *et al.*, 1997; Gotoh *et al.*, 1998b). MexCD-OprJ is still distinguishable from MexAB-OprM, however, by the latter's inability to export additional β-lactams such as carbenicillin and aztreonam (Srikumar *et al.*, 1997; Gotoh *et al.*, 1998b). Indeed, *nfxB* strains are hypersusceptible to β-lactams such as carbenicillin (Hirai *et al.*, 1987; Srikumar *et al.*, 1997; Gotoh *et al.*, 1998b), apparently as a result of the reduced expression of MexAB-OprM in these mutants (Gotoh *et al.*, 1998a). The observation that *nfxB* mutants are hypersusceptible to aminoglycosides (Hirai *et al.*, 1987; Poole *et al.*, 1996a), a major substrate for the MexXY-OprM MDR efflux system (see below), suggests that this efflux system, too, may be down regulated in *nfxB* mutants. The coordinated expression of MDR efflux systems in *P. aeruginosa*, with increases in one compensated for by decreases in another also extends to additional MDR efflux systems in this organism (Li et al., 2000a), reflecting, perhaps, a need to retain only a certain level efflux capability at any given time.

Two classes of *nfxB* mutants have been described, expressing moderate (type A) or high (type B) levels of the efflux system, with resistance levels correlating with efflux gene expression (Masuda *et al.*, 1996). The observation that the original *nfxB* mutant was not resistant to tetracycline or chloramphenicol (Hirai *et al.*, 1987), which is only seen in type B *nfxB* strains (Masuda *et al.*, 1996), suggests that these agents are poor substrates for this efflux system. Thus, comparatively high level expression is necessary for significant drug efflux and observable resistance.

Little is known about the organization and assembly of the MexCD-OprJ MDR efflux system. Topological analysis of MexD has confirmed that as with MexB, MexD possesses 12 membrane-spanning helices with large periplasmic loops of ca. of ca. 300 residues occurring between helices 1 and 2, and 7 and 8 (Gotoh *et al.*, 1999). Moreover, Srikumar *et al* (1997) and Gotoh *et al* (1998a) have demonstrated that OprM can functionally replace OprJ (and vice versa). The MexCD components are also able to function with the *E. coli* TolC protein (Srikumar *et al.*, 1998) and OprM has recently been shown to replace OprN to produce a functional MexEF-OprM chimera (Maseda *et al.*, 2000). Thus, there is substantial flexibility in terms of recruitment of the outer membrane constituents by the inner-membrane and periplasmic efflux components of these tripartite efflux pumps. In contrast, exchanging the inner membrane or periplasmic components of MexAB-OprM with their MexCD-OprJ counterparts failed to yield functional export systems (Yoneyama *et al.*, 1998). Thus, a functional complex is restricted to cognate inner membrane and periplasmic components, due presumably to the specificity of MexC interaction with MexD and MexA interaction with MexB.

MexEF-OprN

The MexEF-OprN system is apparently quiescent in wild type cells, at least under the usual laboratory growth conditions (Köhler *et al.*, 1997a), but is expressed in *nfxC* type multidrug resistant strains isolated in vitro (Fukuda *et al.*, 1990; Masuda *et al.*, 1995; Köhler *et al.*, 1997a) and in the clinic (Fukuda *et al.*, 1995; Jalal *et al.*, 2000). Generally selected as fluoroquinolone-resistant mutants (Fukuda *et al.*, 1990; Masuda *et al.*, 1995), *nfxC* mutants have also been isolated on media containing tetracycline or chloramphenicol (Jalal *et al.*, 1999). *nfxC* mutants display resistance to fluoroquinolones, chloramphenicol, trimethorpim and the carbapenem imipenem (Fukuda *et al.*, 1990; Köhler *et al.*, 1997a). The observed hypersusceptibility to β-lactams and aminoglycosides (Fukuda *et al.*, 1990), a phenotype shared with *nfxB* strains (see above), may result from decreased expression of MexAB-OprM and MexXY-OprM in these mutants. Intriguingly, resistance to imipenem in *nfxC* strains results not from MexEF-OprN expression (Köhler *et al.*, 1997a) but the concomitant decrease in outer membrane protein OprD in these mutants (Fukuda *et al.*, 1990; Masuda *et al.*, 1995). OprD is an imipenem channel and a primary route of entry of this antibiotic in *P. aeruginosa* (Trias and Nikaido, 1990) whose absence is often seen in imipenem-resistant strains of *P. aeruginosa* (Ballestero *et al.*, 1996; Kohler *et al.*, 1999b). The nature of mutation(s) leading to MexEF-OprN hyperexpression has yet to be elucidated although it is dependent upon the product of the *mexT* gene located upstream of *mexEF-oprN*. MexT is a positive regulator of *mexEF-oprN* expression (Köhler *et al.*, 1999a; Ochs *et al.*, 1999) that is also responsible for the decrease in OprD expression in *nfxC* strains (Köhler *et al.*, 1999a;

Ochs *et al.*, 1999). While the cloned *mexT* gene reduces *oprD* gene expression (ca. 2-fold), suggesting that it acts at the level of *oprD* transcription (Köhler *et al.*, 1999a; Ochs *et al.*, 1999), it appears that MexT also acts posttranscriptionally in downregulating OprD production (Köhler *et al.*, 1999a).

MexXY-OprM

Unlike the aforementioned efflux operons, the recently described *mexXY* system (Mine *et al.*, 1999) (also called *amrAB* [Westbrock-Wadman *et al.*, 1999]) lacks a linked outer membrane gene (Mine *et al.*, 1999), reminiscent of the *acrAB* MDR efflux operon of *E. coli* whose outer membrane gene, *tolC*, is also located elsewhere on the chromosome (Ma *et al.*, 1993; Ma *et al.*, 1994; Fralick, 1996). MexXY apparently utilizes OprM as its outer membrane constituent (Aires *et al.*, 1999; Mine *et al.*, 1999; Masuda *et al.*, 2000), consistent with an earlier observation that OprM still functions in efflux-mediated MDR in strains lacking MexAB (Yoneyama *et al.*, 1997; Zhao *et al.*, 1998b). As outer membrane efflux proteins are not functional in the absence of their RND-MFP counterparts (Wong *et al.*, 1997), this was interpreted as OprM operating as the outer membrane component of additional efflux systems (Zhao *et al.*, 1998b). TolC, too, acts as the outer membrane component of several 3-component pumps responsible for exporting uncouplers (Nikaido, 1998), colicin V (Hwang *et al.*, 1997) and hemolysin (Wandersman and Delepelaire, 1990). Strains deleted for *mexXY* show increased susceptibility to aminoglycosides, as well as tetracycline and erythromycin (Aires *et al.*, 1999), indicating that this efflux system contributes to the intrinsic resistance of *P. aeruginosa* to these agents. This resistance is, however, dependent upon induction of MexXY in wild type strains by these agents (Masuda *et al.*, 2000). While the cloned genes also promote resistance to fluoroquinolones (Aires *et al.*, 1999; Mine *et al.*, 1999), indicating that they are accommodated by MexXY-OprM, this efflux system does not contribute to intrinsic resistance to these agents. Hyperexpression of MexXY/AmrAB is seen in a number of so-called impermeability type aminoglycoside-resistant strains of *P. aeruginosa*, and elimination of *amrB* (*mexY*) compromises this resistance, confirming the role of AmrAB/MexXY in the aminoglycoside resistance of these mutants (Westbrock-Wadman *et al.*, 1999). Recently, a mutant constitutively expressing MexXY was reported which demonstrated decreased susceptibility to fluoroquinolones as well as aminoglycosides, indicating that this efflux systems does, indeed, promote fluoroquinolone resistance in *P. aeruginosa* (Masuda *et al.*, 2000). Its failure to facilitate intrinsic resistance to fluoroquinolones results from the inability of fluoroquinolones to induce MexXY expression in wild type cells of *P. aeruginosa* (Masuda *et al.*, 2000). A gene, *mexZ* (also called *amrR* [Westbrock-Wadman *et al.*, 1999]), has been identified upstream of *mexXY* and apparently encodes a repressor of *mexXY*/*amrAB* expression (Aires *et al.*, 1999; Westbrock-Wadman *et al.*, 1999). Deletion of this gene enhances *amrAB*/*mexXY* transcription, although a Δ*amrR* strain is not aminoglycoside-resistant (Westbrock-Wadman *et al.*, 1999), presumably because the required outer membrane component is not hyperexpressed. Thus, aminoglycoside resistance dependent upon AmrAB hyperexpression in impermeability type mutants relies on mutations in genes in addition to or besides *amrR*.

Others

A number of genes encoding homologues of the Mex multidrug efflux systems are readily identifiable in the recently completed *P. aeruginosa* genome sequence (Stover *et al.*, 2000). Two putative three-gene operons encoding candidate MFP-RND-OMF multidrug efflux systems (pa3523-pa3522-pa3521 and pa4206-pa4207-pa4208) and three putative two-gene operons encoding MFP-RND components only (pa1435-pa1436, pa3677-pa3676 and pa4374-pa4375) have been identified (Table 1). Whether or not any of these function in multidrug efflux, the latter systems will require an OMF component. Interestingly, a recent study has shown that a strain deleted for *oprM* is more susceptible to fluoroquinolones and tetracyclines than is a Δ*mexB* Δ*mexXY* double knockout, indicating that OprM contributes to resistance to these agents independent of MexAB-OprM and MexXY-OprM (Poole, K. and Srikumar, R., 2000). Perhaps OprM functions with the MFP-RND products of one of the aforementioned two-gene operons in forming yet another multidrug efflux system.

Multidrug Efflux Pumps and Fluoroquinolones

An interesting feature of the RND-MFP-OMF MDR pumps in *P. aeruginosa* is their contribution to acquired resistance to fluoroquinolones (Poole, 2000). Indeed, there are numerous reports of in vitro- (Rella and Haas, 1982; Hirai *et al.*, 1987; Robillard and Scarpa, 1988; Legakis *et al.*, 1989; Celesk and Robillard, 1989; Fukuda *et al.*, 1990; Radberg *et al.*, 1990; Lei *et al.*, 1991; Masuda and Ohya, 1992) and in vivo- (Piddock *et al.*, 1992; Yoshida *et al.*, 1994) selected fluoroquinolone resistant strains demonstrating a multidrug resistance phenotype. Originally attributed to reduced outer membrane permeability, owing to the decreased drug accumulation and overexpression of novel outer membrane proteins of ca. 50 kDa molecular mass (Legakis *et al.*, 1989; Celesk and Robillard, 1989; Fukuda *et al.*, 1990; Masuda and Ohya, 1992; Masuda *et al.*, 1995), it is now clear that these represented mutants hyperexpressing MDR efflux systems. According to a recent study, in fact, the bulk of in vitro-selected fluoroquinolone-resistant strains hyperexpress MexCD-OprJ or MexEF-OprN (Köhler *et al.*, 1997b). Indeed, while target site (i.e. topoisomerase) mutations typically prevail in fluoroquinolone-resistant bacteria (Köhler and Pechere, 1998), in vitro-selected fluoroquinolone- resistant strains of *P. aeruginosa* typically exhibit the efflux-mediated MDR phenotype (Yoshida *et al.*, 1990; Iyobe *et al.*, 1991; Jakics *et al.*, 1992; Köhler *et al.*, 1997b). Moreover, eliminating these efflux mechanisms by mutation both compromises resistance due to target site mutations and obviates selection of fluoroquinolone resistance in vitro (Lomovskaya *et al.*, 1999). Given the significance of efflux mechanisms in fluoroquinolone resistance in *P. aeruginosa*, then, it is not surprisingly that MDR efflux pumps are an

attractive target for therapeutic intervention (Renau *et al.*, 1999; Lomovskaya *et al.,* 2001).

Burkholderia spp

Originally described as the causative agent of soft rot in onions, *B. cepacia* is increasingly important as an opportunistic human pathogen, particularly in patients with cystic fibrosis or chronic granulomatous disease (Govan *et al.*, 1996; Quinn, 1998). As with many other non-fermenting Gram-negative bacilli, the organism is intrinsically resistant to multiple antimicrobial agents and many clinical strains exhibit multidrug resistance (Govan *et al.*, 1996) (Quinn, 1998). Although the outer membrane barrier likely plays a significant role in this (Aronoff, 1988), MDR efflux pumps have also been implicated. CeoAB-OpcM (GenBank accession number U97042) is an RND-MFP-OMF type MDR efflux system (Burns *et al.*, 1996; J. Burns *et al.*, unpublished) that was first identified as a determinant of chloramphenicol resistance in a clinical strain (Burns *et al.*, 1989). Providing resistance to trimethoprim and fluoroquinolones in addition to chloramphenicol, it is unclear whether this efflux system is only expressed in mutant strains or is also expressed constitutively, where it would contribute to intrinsic antimicrobial resistance. A regulatory gene has not been reported for this efflux system, although salicylate induction of multidrug resistance in *B. cepacia* (Burns and Clark, 1992) is suggestive of a *mar* locus in this organism (see above). A RND-MFP-OMF homologue, AmrAB-OprA, has recently been described in *B. pseudomallei* (Moore *et al.*, 1999), the highly antibiotic resistant (Moore *et al.*, 1999; Simpson *et al.*, 1999) causative agent of melioidosis (Dance, 1991). Also a MDR transporter, this system exports and provides resistance to aminoglycosides and macrolides.

Stenotrophomonas maltophilia

An increasingly important nosocomial pathogen, particularly in debilitated or immunosuppressed individuals, *S. maltophilia* is resistant to multiple antimicrobial agents (Quinn, 1998; Denton and Kerr, 1998). Multidrug resistance attributable to efflux has been reported in this organism (Alonso and Martinez, 1997; Zhang *et al.*, 2000) and, indeed, homologues of the MexB and OprM components of the MexAB-OprM MDR efflux systems of *P. aeruginosa* have been identified in clinical MDR strains of *S. maltophilia* (Zhang *et al.*, 2000). As with *P. aeruginosa*, fluoroquinolones readily select MDR strains in vitro (Lecso-Bornet *et al.*, 1992; Zhang *et al.*, 2000) although other agents such as tetracycline and chloramphenicol also yield MDR strains (Alonso and Martinez, 1997; Zhang *et al.*, 2000). Recently, a RND-MFP-OMF type efflux system, SmeABC, was identified in this organism (GenBank accession number AF173226). The SmeC gene product cross-reacts with antibodies to the *P. aeruginosa* OMF, OprM, and can functionally replace OprM in an OprM-deficient mutant (L. Zhang, X.-Z. Li and K. Poole, unpublished). Expression of *smeABC* is under the control of the products of the linked *smeRS* genes that encode a classical phosphorylation-dependent two-component regulatory system (L. Zhang, X.-Z. Li and K. Poole, unpublished). A second RND-MFP-OMF type MDR efflux system has been identified in *S. maltophilia*, encoded by the *smeDEF* genes (Alonso and Martinez, 2000). This appears to be the efflux system responsible for the multidrug resistance of previously described MDR strains of *S. maltophilia* (Alonso and Martinez, 1997).

Pseudomonas putida

P. putida is not generally a human pathogen, although the organism has gained a degree of notoriety as a result of the ability of certain strains to tolerate high concentrations of toluene (Inoue and Horikoshi, 1989). In many instances this results from solvent efflux (Isken and de Bont, 1996) by homologues of the RND-MFP-OMF MDR efflux systems. The first to be reported, the SrpABC efflux system of *P. putida* S12, accommodates a variety of organic solvents although no medically relevant antimicrobial agents (Kim *et al.*, 1998; Kieboom *et al.*, 1998a). In contrast, the MepABC MDR efflux system of *P. putida* KT2442 accommodates both organic solvents and antimicrobials (Fukumori *et al.*, 1998), reminiscent of the MDR efflux systems of *P. aeruginosa* (see above). Indeed, the pattern of antimicrobial resistance afforded by MepABC is very reminiscent of MexAB-OprM, the efflux system displaying the highest degree of amino acid sequence homology to the Mep proteins (66-70 % identity) (Fukumori *et al.*, 1998). A related system from *P. putida* DOT-T1E, TtgABC, affords resistance to toluene as well as several antimicrobials (Ramos *et al.*, 1998), a pattern also reminiscent of the *P. aeruginosa* MDR efflux systems. Finally, a second toluene efflux system of the RND-MFP-OMF type has recently been described in *P. putida* DOT-T1E. Encoded by the *ttgDEF* genes that are linked to the *tod* genes of toluene metabolism, this efflux system facilitates toluene tolerance but not resistance to antimicrobials (Mosqueda and Ramos, 2000). As with the *srpABC* genes (Kieboom *et al.*, 1998b), the *ttgABC* efflux genes are inducible by aromatic hydrocarbons (Mosqueda and Ramos, 2000), suggesting that solvent export is a primary function of these two efflux systems. (Ramos *et al.*, 1998).

Conclusions

The characteristic intrinsic antimicrobial resistance of *P. aeruginosa* owes much to the presence of tripartite MDR efflux systems in this organism, as does the acquired fluoroquinolone-resistance of clinical strains. Nonetheless, efflux substrates extend well beyond clinically-relevant agents, signifying that antimicrobial efflux and resistance is not the intended function of these systems. Using DNA microarray technology and proteomics it should soon be possible, however, to identify additional genes/proteins that are co-expressed with MDR efflux genes/components, and their identification might suggest a function. In the meantime, these efflux pumps are arguably good therapeutic targets for countering intrinsic and acquired antimicrobial resistance in *P. aeruginosa* and other multiply resistant Gram-negative pathogens.

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Note Added in Proof

A recent paper (Pai *et al.,* 2001. Antimicrob. Agents Chemother. 45: 480-484) confirms that loss of OprD is the primary determinant of carbapenem resistance in *P. aeruginosa* although meropenem resistance owing to MexAB-OprM hyperexpression was also noted. Using well-defined efflux mutants, the export of ß-lactams by MexAB-OprM, MexCD-OprJ and MexXY-OprM was confirmed, although MexXY-OprM was a much less effective exporter of ß-lactams than the other two, and MexAB-OprM was superior to MexCD-OprJ as regards export of penicillins and oxacepehms (Masuda *et al.,* 2000. Antimicrob. Agents Chemother. 44: 3322-3327). The role of MexCD-OprJ and MexEF-OprN, but not MexXY-OprM in the export of and resistance to triclosan, a fatty acid biosynthesis inhibitor with broad-spectrum antimicrobial activity has also been reported (Chuanchuen *et al.,* 2001. Antimicrob. Agents Chemother. 45: 428-432). *In vivo* selection of MDR strains of *P. aeruginosa* hyperexpressing MexCD-OprJ or MexEF-OprN has been reported in an acute pneumonia rat model of infection (Join-Lambert *et al.,* 2001. Antimicrob. Agents Chemother. 45: 571-576). Intriguingly, such mutants arose rapidly in the absence, as well as in the presence of antibiotic selection. The noted difficulty in selecting MexEF-OprN-hyperexpressing *nftC* mutants from certain strains of *P. aeruginosa* was recently explained by the observation that several so-called wild type strains carry mutations in the *mexT* gene required for *mexEF-oprN* hyperexpression (Maseda *et al.,* 2000. FFMS Microbiol. Lett. 192: 107-112).

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